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Is Required for the Proliferation of Melanoblasts in the Mouse Embryo

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The development of neural crest-derived melanocytes, as well as haematopoietic and germ cells, is affected by mutations of the *Kit* and *Mgf* genes, which lead to dominant spotting (*W*) or steel (*Sl*) phenotypes. *Mgf* codes for the ligand of the receptor tyrosine kinase encoded by the *Kit* locus. $Kit^{W\text{-}v}$, a point mutation exerting a dominant negative effect, causes a substantial reduction in tyrosine kinase activity of the Kit receptor and leads to a characteristic pigmentation phenotype, namely dilute coat colour and a white ventral and head spot with reduced pigmentation of the feet and tail in the heterozygous animal, as well as slight anaemia. Homozygous animals lack coat pigmentation and are severely anaemic and infertile. Dct is a marker for cells of the melanoblast lineage. In order to study these cells in detail we have generated transgenic mouse lines carrying the *lacZ* reporter under the control of the *Dct* promoter and have used the embryonic expression of the reporter to identify early melanoblasts before they begin to produce pigment. Our transgenic lines have simplified the study of melanoblasts in the mouse embryo, and by crossing our mice with $Kit^{W\text{-}v}$ mutants we have been able to identify the midgestation stages at which melanoblasts rely critically on Mgf/Kit interactions. We conclude that the survival of immature melanoblasts depends crucially upon Kit signalling up until E11, and later in development Kit plays a vital role in melanoblast proliferation. Our data do not describe a dependence upon Kit for melanoblast migration or differentiation.

INTRODUCTION

Melanocytes, the highly specialised melanin-producing cells of the hair, skin, and choroid, arise in the trunk neural crest at the time of closure of the neural tube. Their precursors, melanoblasts, migrate from here on a dorsolateral pathway, between the dermamyotome of the somites and the overlying ectoderm to their eventual destination in the basal layer of the epidermis or the hair follicle (reviewed by Erickson, 1993). Other neural crest-derived melanoblasts migrate to sites around the eye and to the stria vascularis of the inner ear. A second population of pigment cells differentiates from neuroectoderm *in situ* to become the retinal pigment epithelium of the eye.

The development of neural crest-derived melanocytes is affected by mutations of the *Kit* and *Mgf* genes, which lead to dominant spotting (*W*) or steel (*Sl*) phenotypes, respectively. These mutations also affect germ cells and haematopoietic cells. Mice homozygous for either Kit^{W} or Mgf^{Sl} mutations show a similar phenotype: a lack of coat pigmentation, sterility, and a deficiency of erythrocytes and mast cells. The gene products encoded at these loci have complementary sites of action. Grafting experiments have shown that the *Mgf* product affects the microenvironment which supports these cell types, whilst the product of the *Kit* locus is cell autonomous and acts within the stem cell populations (Mayer and Green, 1968). The identification of the genes at these loci confirmed the hypothesis that the *Kit* and *Mgf* loci encode a receptor and its ligand, respectively. The *Kit* locus encodes the receptor tyrosine kinase Kit, a proto-oncogene belonging to the same family as the Pdgf and Csf-1 receptors (Chabot *et al.*, 1988; Geissler *et al.*, 1988). The *Mgf* locus encodes mast cell growth factor (also known as steel factor or stem cell factor), the ligand for the Kit receptor, which exists in both membrane-bound and soluble forms (Copeland *et al.*, 1990; Huang *et al.*, 1990; Zsebo *et al.*, 1990; Williams *et al.*, 1990).

Numerous mutant alleles exist at the *Kit* locus. The $Kit^{W\text{-}v}$ mutation, a substitution of a threonine for methionine residue at amino acid position 660 of the receptor

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(within the ATP-binding region of the kinase domain) (Hanks *et al.*, 1988), substantially reduces the tyrosine kinase activity of the mutant protein compared to the wild-type receptor (Nocka *et al.*, 1989; Reith *et al.*, 1990). Heterozygous animals display a characteristic pigmentation phenotype—dilute coat colour with a white belly and head spot and reduced pigmentation of the feet and tail. They are also slightly anaemic though remain fertile. Homozygous animals have an almost complete lack of coat pigmentation, are severely anaemic, and are infertile. The unpigmented skin and hair follicles of *W* mutant animals contain no melanocytes, presumably due to the failure of melanoblasts to differentiate, migrate, survive, or proliferate in the absence of the normal Mgf/Kit interaction. Previous studies have shown that survival of melanoblasts requires membrane-bound Mgf and the presence of functional Kit (Steel *et al.*, 1992; Cable *et al.*, 1995; Wehrle-Haller and Weston, 1995). Those melanoblasts which have differentiated from the neural crest and begun their migration fail to survive, and by the 12th day of gestation have almost completely disappeared.

Experiments injecting monoclonal antibodies to block Kit receptor activity show that Kit is required for melanoblast survival or differentiation in the early embryo, for proliferation and entry of these cells into the epidermal tissue around E14, and finally in postnatal life for melanocyte activation in developed hair follicles (Nishikawa *et al.*, 1991; Yoshida *et al.*, 1993, 1996). Manipulation of embryos *in utero*, however, may affect subsequent development and survival. In addition, the conclusions of these experiments were drawn from the analysis of pigmentation patterns in the adult animal. Several groups have chosen, instead, to study the dependency of melanoblasts on Mgf/Kit interactions through use of cultured neural crest tissue (for example, Murphy *et al.*, 1992; Morrison-Graham and Weston, 1993; Reid *et al.*, 1995; Langtimm-Sedlak *et al.*, 1996). Whilst this approach is powerful and has led to advances in our understanding of the conditions required for melanocyte differentiation from nascent neural crest cells, spatial and environmental cues for development are lost. We describe here experiments which allow visualisation of the temporal and spatial development of melanoblasts under conditions of normal and abnormal Kit expression during embryonic development. In addition, we have examined the melanoblasts of heterozygous mutant embryos, in which these cells escape death at E11, to reveal later requirements for Kit.

At least three melanogenic enzymes are involved in the production of black/brown eumelanin, namely tyrosinase and the tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2, or dopachrome tautomerase, DCT). Tyrosinase (EC 1.14.18.1) is the key enzyme in the melanogenic pathway and its action is rate limiting. TRP-1 appears to be 5,6-dihydroxyindole 2-carboxylic acid (DHICA) oxidase, which acts in the conversion of DHICA into 5,6-quinone 2-carboxylic acid (Kobayashi *et al.*, 1994), and mutations in the gene (*brown*) alter the colour of eumelanin produced from black to brown. DCT (EC 5.3.2.3) catalyses the conversion of DOPAchrome to DHICA. Mutations at this locus also affect the quality of eumelanin formed, giving rise to the production of a dark grey/brown eumelanin compared with the wild-type black pigment. To date, only three mutant alleles of *Dct* have been described. The original slaty mutation (Dct^{slt}) has a partial loss of enzymatic function, and a second mutant allele, slaty-2J ($Dct^{slt\text{-}2J}$), is phenotypically identical. The slaty-light ($Dct^{Slt\text{-}lt}$) mutation is more severe and acts in a semidominant fashion (Budd and Jackson, 1995).

We have previously identified Dct as a marker for cells of the melanoblast lineage (Steel *et al.*, 1995). In order to study these cells in more detail we have generated transgenic mouse lines carrying the reporter gene *lacZ* under the control of the *Dct* promoter and have used the embryonic expression of the reporter to identify early melanoblasts before the production of pigment begins. We have also observed expression of the transgene in the telencephalon, as previously reported for Dct (Steel *et al.*, 1992; Pavan and Tilghman, 1994). Further, we have examined the effect of the $Kit^{W\text{-}v}$ mutation on the expression of the reporter to enhance our understanding of the requirement for the Mgf/Kit interaction. The telencephalon expression is Kit independent. In melanoblasts the Mgf/Kit interaction is required early as a survival signal, but later in development it may promote melanoblast proliferation.

MATERIALS AND METHODS

Transgenic Mice

The construct used for microinjection into fertilised CBA × C57BL/6 F1 oocytes was made using the mouse *Dct* (TRP-2) promoter from −3181 to +445 (numbering as in Budd and Jackson, 1995) cloned into vector pBSK+/− adjacent to the 3.7-kb *Hin*dIII/*Bam*HI fragment containing the *lacZ* gene and SV40 splice and polyadenylation signals (Lowings *et al.*, 1992). Independent transgenic lines were derived from five founder animals shown to be carrying the transgene.

Genotyping

Identification of transgenic animals and embryos was by PCR of tail biopsy or extraembryonic membrane DNA using the primers 5′-GAATTATTTTGATGGCGTTA-3′ and 5′-CGCTGATTTCTGTAGTCGGTT-3′, which amplify a 252-bp fragment within the *lacZ* gene. A PCR assay (as described by Cable *et al.*, 1995) was used to identify $Kit^{W\text{-}v}$ mutants. Amplification using the primers 5′-ACACGGCTTTACCTCCCACC-3′ and 5′-AAAGAGAGGCCCTAATGTCG-3′ yielded a 105-bp product. The $Kit^{W\text{-}v}$ mutation resulted in the transition of a C to a T at nucleotide position 2007, creating an *Nsi*I site. Digestion of the mutant PCR product cleaved the 105-bp fragment into 84- and 21-bp fragments; the wild-type PCR product was not digested by *Nsi*I.

Analysis of Embryos

The time of gestation was calculated by taking noon of the day of detection of a vaginal plug as embryonic day 0.5 (E0.5) and also by noting external appearance of the embryo (according to Kaufman, 1992). Expression of the transgene was detected by staining staged embryos overnight in XGal buffer. Embryos were dissected free of extraembryonic membranes (which were retained for genotype analysis) then fixed in 4% paraformaldehyde at 4°C for 30 min

to 2 h, depending on their developmental stage, washed in detergent wash (2 mM $MgCl_2$, 0.05% BSA, 0.1% sodium deoxycholate, 0.02% NP-40 in 0.1 M sodium phosphate buffer, pH 7.3), and stained overnight in detergent wash containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 0.085% NaCl with 0.1% XGal at 37°C. The staining reaction was stopped by washing the embryos in detergent wash then in phosphate-buffered saline. The embryos were postfixed overnight in 4% paraformaldehyde at 4°C.

Staining for lacZ in Adult Tail Skin

Whole skin was peeled from mouse tail, pinned out flat, and incubated for 2 h in 2 M sodium bromide at 37°C. The epidermis was peeled away from the dermis, fixed for 1 h in 4% paraformaldehyde, then stained for *lacZ* activity using the same protocol as for embryos. Intact skin samples were fixed and stained in the same way as was embryonic tissue.

Histology

Stained and postfixed tissue was washed overnight in Vibratome solution (20% sucrose, 16% bovine serum albumin, 0.5% gelatin, 0.05% sodium azide in PBS), then embedded in the same solution using 5% glutaraldehyde as the cross-linking agent. Vibratome sections were mounted in aqueous mountant (20% sucrose, 16% gelatin, 0.05% sodium azide in PBS).

RESULTS

Analysis of Dct-lacZ in Wild-Type Embryos

Five transgenic mouse lines were established following microinjection of the *Dct-lacZ* transgene. We analysed the expression of the transgene by staining embryos with XGal between embryonic stages E9 to E16.5. Each line showed a similar pattern of *lacZ* expression at every stage examined, though with varying intensity of staining. The line showing strongest staining in melanoblasts, A12, was chosen for detailed analysis.

The *Dct* promoter is active from E9; no staining can be detected in embryos younger than this. In transgenic embryos *lacZ* expression is seen initially in the eye at E9 then later in the telencephalon (Fig. 1). The eye forms from the optic vesicle, which is itself derived from an outgrowth of the diencephalon. During day 9 of gestation the vesicle invaginates to become the bilayered optic cup, the neuroectodermal outer layer of which differentiates into a single layer of pigmented epithelium, the retinal pigment epithelium (RPE). We detect transgene expression from E9 (17–20 pairs of somites) in the presumptive RPE. Expression is first seen as a crescent of staining at the posterior part of the optic vesicle, which represents the site of earliest differentiation of the simple neuroepithelium into RPE. By E10.5 expression has spread throughout the RPE, characterising differentiation of this tissue (Fig. 2A). Expression in the RPE persists throughout all stages examined and is still detectable by staining after melanin deposition. This pattern is in accord with that previously described by Steel *et al.* (1992). The *lacZ* expression seen caudally in the late E9.5 embryo (Fig. 1) is the beginning of the neural expression discussed below.

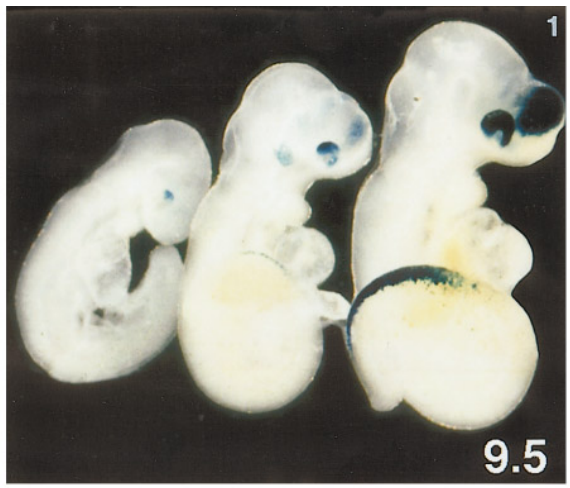
Previously, expression of *Dct* had been noted in the telencephalon at E12.5. Our analysis now shows that the gene is active 3 days before that. We find XGal staining in the future forebrain from E9.5 (20–25 pairs of somites) (Fig. 1). By this stage the cephalic neural folds have fused and the telencephalic vesicles are becoming evident. Transgene expression in the telencephalon continues until at least E17.5. Development of the olfactory region of the brain begins at about E13, and by E14 the olfactory lobes become easily distinguished from the remainder of the neophallial cortex. We detect XGal staining in the olfactory bulbs at E14.5 (Fig. 2C), which persists through E15.5 and E16.5.

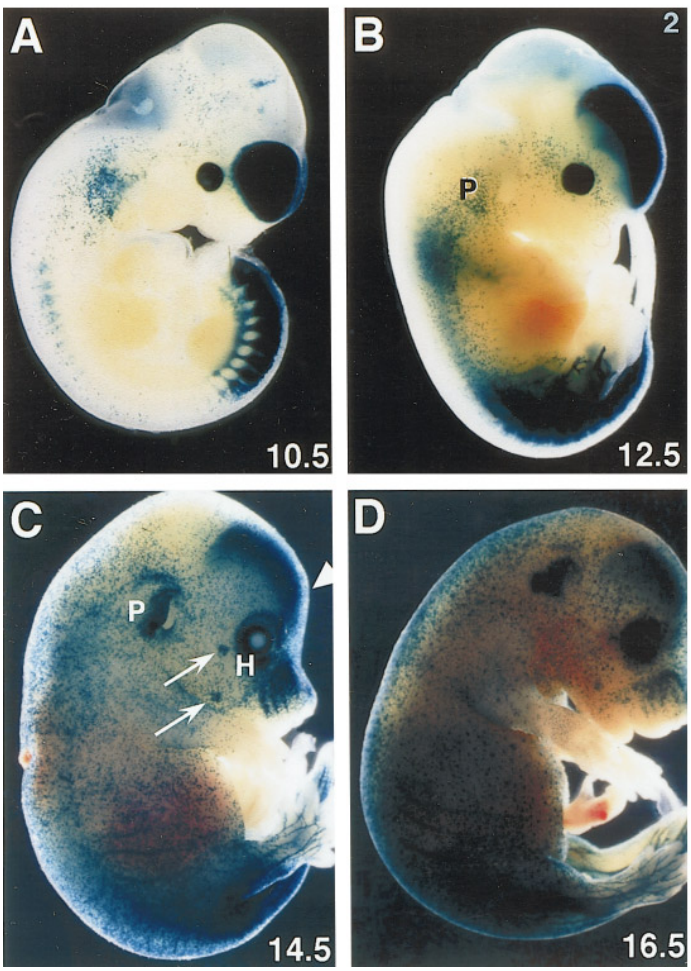
XGal staining is detected dorsal and lateral to the future

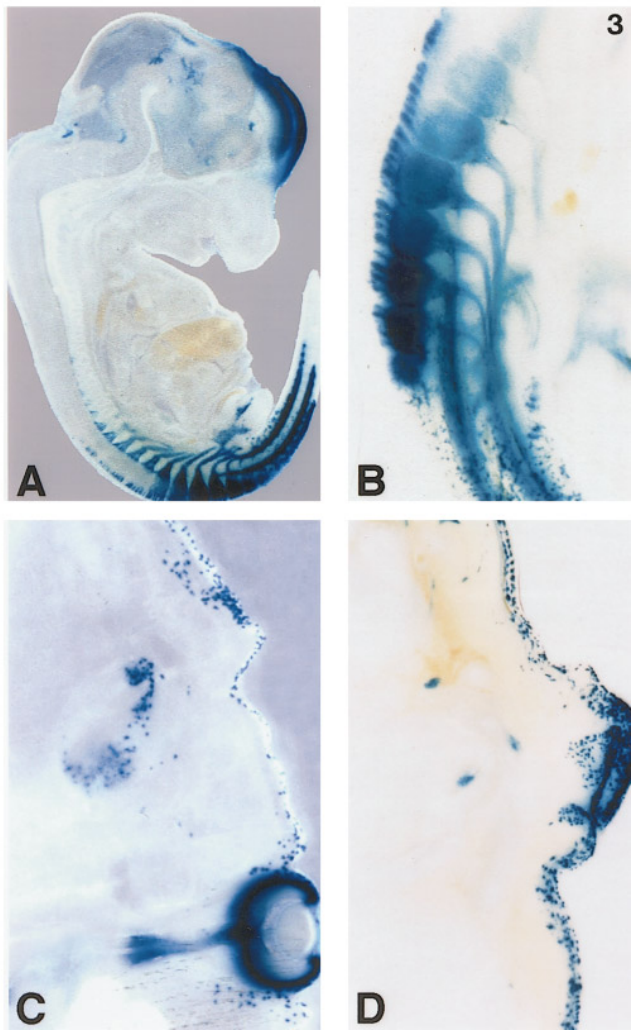
FIG. 1. XGal-stained *Dct-lacZ* transgenic embryos: E9, E9.5, and late E9.5. Transgene expression is observed in the early E9 embryo (left) in the optic cup only. At a slightly later developmental stage (centre) staining is observed in the newly forming telencephalon and is beginning to become visible in the caudal neural tube. By late E9.5 (right) strong staining is visible in the telencephalon. Caudal expression has become much stronger and is now apparent in condensations of cells which will become dorsal root ganglia.

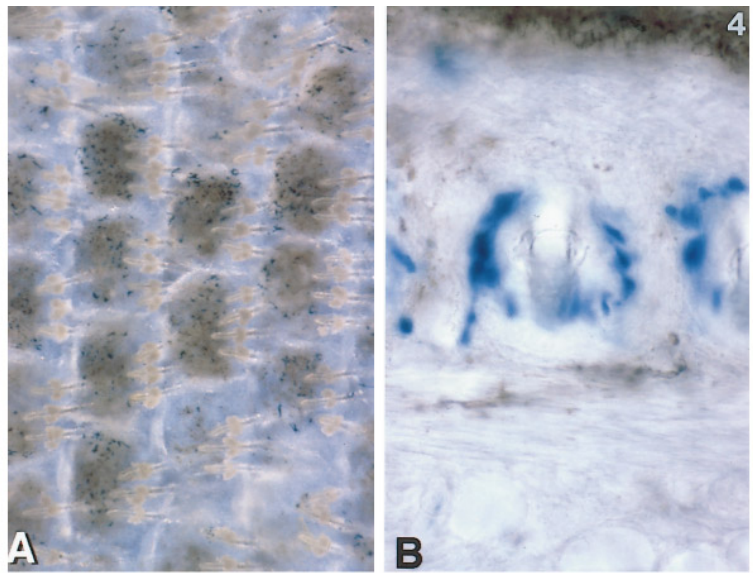
FIG. 2. XGal-stained *Dct-lacZ* transgenic embryos: (A) E10.5, (B) E12.5, (C) E14.5, and (D) E16.5. (A) At E10.5 melanoblasts are sparsely scattered over the dorsolateral aspect of the trunk and tail neural tube. In the head, melanoblasts are present over the mesencephalon, extending forward towards the eye. (B) By E12.5 melanoblasts surround the eye and are present in a broad band curving back beyond the ear to the shoulder region. Melanoblasts also occupy the mesenchyme lateral to the spinal cord well into the tail. The ventrum shows no labelled cells. P, pinna. (C) At E14.5 almost the entire surface of the embryo is well populated by XGal-staining melanoblasts, with a particularly high concentration of stained cells in the pinna (P). The vibrissae around the mouth and the follicles of other sensory hairs of the face (arrows) contain large numbers of melanoblasts. A high concentration of labelled cells encircles the eye, the darker region immediately below the eye being the Harderian gland (H). By E14.5 the stained olfactory bulb (arrowhead) can be distinguished from the telencephalon. Melanoblasts have populated the hind and forelimbs, but not the feet. (D) E16.5. Melanoblasts have just begun to populate the feet, but not the ventral surface of the limbs.

FIG. 3. Vibratome sections of XGal-stained *Dct-lacZ* transgenic embryos: (A) E12.5, (B) E14.5, (C) E12.5, and (D) E15.5. (A) Sagittal section (100 μm) showing forebrain and dorsal root ganglia (DRG) expression in E12.5 embryo. (B) E14.5 sagittal section, 100 μm. Close up of DRG at sacral level showing ventral roots converging to form main caudal nerves. (C) E12.5, transverse section, 100 μm. Strong transgene expression is seen in the RPE and optic stalk. Labelled melanocytes are also detected in the choroid and Harderian gland associated with the eye, in the developing inner ear, and in the skin. (D) E15.5, transverse section, 200 μm. Melanocytes are present in the skin, being particularly densely packed in the pinna, and are visible in the inner ear.









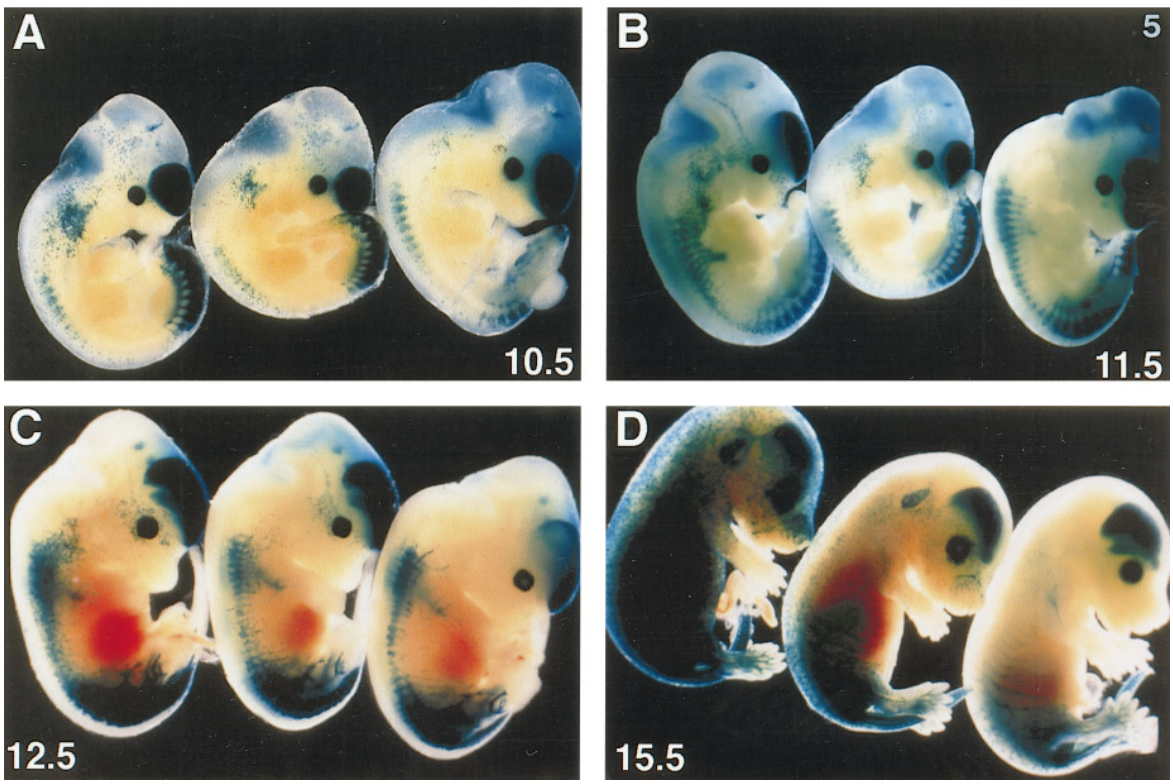


FIG. 4. Transgene expression in adult skin. (A) XGal staining of the underside of stained transgenic tail epidermis, separated from the dermis, reveals numerous melanocytes associated with each pigmented scale. (B) A section cut through XGal-stained intact transgenic skin showing the clustering of melanocytes around the hair follicles. Individual melanocytes can also be seen in the basal layer of the pigmented epidermis.

FIG. 5. XGal staining of $Kit^{W\text{-}v}$ mutant and wild-type *Dct-lacZ* transgenic embryos: Left, +/+ embryo, centre, $Kit^{W\text{-}v}$/+, and right, $Kit^{W\text{-}v}/Kit^{W\text{-}v}$. (A) E10.5. Those regions in which melanoblast density is highest in the +/+ embryo are where melanoblasts are observed in the $Kit^{W\text{-}v}$/+ embryo and, to a much lesser extent, in the $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ embryo. A few melanoblasts can be seen lateral to the trunk spinal cord in the homozygous mutant. (B) E11.5. Heterozygous embryos have a somewhat reduced melanoblast number. Very few melanoblasts can be detected in the homozygous mutant embryo. (C) E12.5. The distribution of stained melanoblasts in the $Kit^{W\text{-}v}$/+ embryo is similar to that seen in the +/+ embryo, but the decrease in numbers is marked. No melanoblasts at all can be seen in the $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ embryo. (D) E15.5. Melanocyte numbers in the heterozygous embryo are now greatly reduced compared to wild type. The accumulation of melanoblasts around the eye is very obvious when compared with the $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ embryo in which XGal staining in the eye is due only to RPE. No neural crest-derived melanocytes can be identified in the $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ embryo.

caudal spinal cord from E9.5 (about 30 pairs of somites) (Fig. 1). Trunk neural crest cells, in addition to giving rise to melanocyte precursors, form neurons and glia of sensory and sympathetic ganglia, Schwann cells, and adrenal chromaffin cells. We cannot detect XGal staining in the adrenal glands, despite this common ancestry of adrenal medullary cells and melanoblasts. By E10.5 there is clear staining of dorsal root ganglia of the trunk and tail and of the caudal spinal nerves (Figs. 2, 3A, and 3B). Authenticity of expression of *Dct* in the telencephalon at E12.5 was previously established by *in situ* hybridisation to mRNA (Steel *et al.*, 1992) and by immunohistochemistry (Pavan and Tilghman, 1994). On the other hand, we have been unable to detect *in situ* hybridisation of *Dct* probes to caudal nerves at E11.5 to E15.5 (data not shown). Furthermore, Pavan and Tilghman (1994) examined transverse sections throughout the length of embryos from E10 to E18.5 by immunohistochemistry and did not detect Dct protein in dorsal root ganglia or nerves (W. Pavan, pers. comm.). It appears that *lacZ* expression at these sites in the five independent transgenic lines examined is not matched by endogenous *Dct* expression.

Neural expression of the transgene is also detected in the optic stalk at E12.5 (Fig. 3C) but, once again, *in situ* hybridisation to this tissue is negative. The optic stalk forms by a narrowing at the proximal end of the optic vesicle and can be considered contiguous with the RPE. It appears that expression of the transgene extends beyond the normal domain of *Dct* in the embryonic eye. In adult mice the RPE expression is maintained, but the adult optic stalk is negative for transgene expression (data not shown).

XGal staining of transgene expression detects individual melanoblasts after they have left the neural crest from as early as E10.5 (Fig. 2A). Melanoblasts are first seen in a diamond-shaped area in the mesenchyme just lateral to the cervical neural crest at about the level of the branchial arches, apparently migrating towards the head. Cranial neural crest is thought not to contribute to the melanoblast lineage, therefore all melanocytes of the head must originate more caudally and migrate in a rostral direction. At E10.5 and E11.5 we see a stream of labelled melanoblasts which appear to be migrating towards the eye and ear (Figs. 2A and 5B). Only a few labelled melanoblasts are observed in trunk and caudal regions of the embryo at E10.5.

Over the next 2 days of development more melanoblasts appear caudally (Fig. 2B). By E12.5 there is a significant increase in the number of labelled cells caudal to the hindlimbs and level with and rostral to the forelimbs. The midtrunk region is noticeably less well populated by melanoblasts and at all levels the dorsal regions contain many more labelled cells than ventral parts. This initial dorsal–ventral difference in melanoblast density and the paucity of melanoblasts in the midtrunk persists until at least E16.5, the latest time at which we are able to stain the embryos (Figs. 2C and 2D). Melanoblasts begin to enter the forelimbs at E12.5 and the hindlimbs a day or 2 later, but they are not found in the feet until E16.5. Melanoblasts are present in the tail from E12.5, but do not appear in the tip even as late as E16.5.

From E12.5 melanoblasts in the head accumulate more densely around the eye, where they contribute to the choroid and the Harderian gland. Melanocytes also contribute to the stria vascularis of the inner ear, and the precursors of these cells can be seen stained with XGal beginning to populate the developing cochlea (Fig. 3C). Whisker follicles are forming at this stage but do not as yet contain melanoblasts. By E14.5 melanoblasts are accumulating in these vibrissae (Fig. 2C). Throughout this period melanoblasts are also observed over the entire surface of the head. Between E12.5 and E14.5 labelled cells also accumulate in the pinna (Figs. 2C and 3D) which in adults is one of the few regions where melanocytes pigment the skin. Perhaps a greater number of melanoblasts is needed to produce pigmented skin than pigmented hair. It is notable that $Kit^{W\text{-}v}$ homozygotes often have pigmented external ears; it seems likely that the high concentration of melanoblasts in the pinnae means that some escape the effects of the mutation.

After E17.5 the development of the skin means that the XGal stain is unable to penetrate, so staining is no longer seen. However, transgene expression can be detected in the melanocytes of the hair follicles and epidermis of pigmented (tail and pinna) skin biopsies from adult transgenic mice (Fig. 4).

Despite the additional neural staining seen in the transgenic embryos, our mice have enabled easy observation of the normal development of melanoblasts during midgestation and have provided spatial and temporal information which is lost using other methods.

Effect of $Kit^{W\text{-}v}$ Mutation on Melanoblast Development

Dct-lacZ transgene expression provides an excellent lineage marker for melanoblasts. We crossed the transgene onto animals carrying the $Kit^{W\text{-}v}$ mutation and used XGal staining to assay the effects on melanoblast development.

Homozygous $Kit^{W\text{-}v}$ mice are infertile, therefore matings were set up between heterozygous animals, one of which contributed the *Dct-lacZ* transgene. No difference in expression of the transgene has been seen whether it was transmitted from the mother or the father. Transgenic embryos that were wild-type, heterozygous, or homozygous for the $Kit^{W\text{-}v}$ mutation were obtained from each litter and were genotyped by PCR analysis of DNA from the extraembryonic membranes. Figure 5 shows for each gestational age a wild-type, heterozygous, and homozygous embryo from within the same litter.

A comparison of wild-type and homozgous $Kit^{W\text{-}v}$ embryos shows that at E10.5 mutant embryos contain fewer melanoblasts, although their spatial distribution over the head and rostral trunk is similar to wild-type. By E11.5 the number of melanoblasts in mutant embryos has greatly decreased. Those which do survive until this stage follow a narrow line curving dorsally and caudally from the eye towards the shoulder region (Fig. 5B). By E12.5 and later no melanoblasts at all can be seen. These observations are con-

sistent with previous reports that Kit provides a survival signal for melanoblasts.

The effect of heterozygosity for $Kit^{W\text{-}v}$ on melanoblasts gives more information as to the role of this receptor tyrosine kinase. As early as E10.5 there is a significant decrease in the number of melanoblasts in heterozygotes (although not as great as in homozygotes). This difference becomes greater as development progresses; the melanoblast numbers in heterozygotes do not increase at the same rate as in wild type. Thus at E11.5, E12.5, and E15.5, although the number of stained cells in heterozygotes increases, the relative number in comparison to wild type becomes more and more striking (Fig. 5). Particularly noticeable is the decrease on the ventral surface, such that the dorsoventral gradient of melanoblast numbers seems to be more pronounced.

DISCUSSION

Dct Expression

The *Dct-lacZ* transgene provides an excellent marker for studying melanocyte behaviour from an early stage during embryogenesis. The transgenic lines described here facilitate the study of differentiation, migration, survival, and proliferation of melanocytes in the mouse embryo, with the advantage that the effects of various mutations may be investigated by crossing the transgene onto mutant animals. Previous studies using *in situ* hybridisation of a *Dct* probe to tissue sections or to whole mounts have been limited in their spatial and temporal range (Cable *et al.*, 1995; Wehrle-Haller and Weston, 1995). Our work permits identification of presumptive neural crest-derived melanoblasts from the time of their appearance at about E10.5 and allows us to follow their migration to the skin up to E16.5. The expression of the transgene is still detected in adult skin and hair follicles.

The *Dct-lacZ* transgene also identifies the neuroectoderm-derived pigment cells of the RPE and refines the timing of expression of *Dct* in the forebrain which had previously been shown by *in situ* hybridisation (Steel *et al.*, 1992) and immunohistochemistry (Pavan and Tilghman, 1994). The strong staining in the lateral ventricles and the olfactory bulbs is similar to the *tyrosinase-lacZ* transgene expression pattern reported by Tief *et al.* (1996b). Although tyrosinase protein is expressed in the forebrain, enzyme activity is not detected (Tief *et al.*, 1996a) and loss of function mutations of tyrosinase (*albino* mutants) show no obvious neurological defects associated with the forebrain. We do not know if the Dct protein detected in the mouse forebrain is active, nor if it has any function there. It is interesting to note that only three mutant alleles of *Dct* (*slaty*) have been described, all of them missense mutations without necessarily a complete loss of Dct activity (Budd and Jackson, 1995). In fact, at least one retains detectable activity (Jackson *et al.*, 1992). A complete knockout of the *Dct* gene may be of interest in defining the role of Dct in pigment synthesis and may clarify whether it is necessary for telencephalon development.

The expression of *Dct-lacZ* in the dorsal root ganglia, optic stalk, and nerves of the abdomen and hindlimb does not correspond, as far as we can detect using *in situ* hybridisation, to authentic expression of *Dct*. As all our transgenic lines show this unexpected pattern it is unlikely that the effect is due to the integration site of the transgene; we have shown by fluorescence *in situ* hybridisation that in at least two of the transgenic lines the transgene maps to different chromosomal locations. It is more likely that the promoter used in the construction of the transgene does not contain all the control elements of the *Dct* gene and that a neural repressor element may operate at some distance from the gene to repress normal expression of *Dct* in the nerves in which we detect lacZ staining. Alternatively, the juxtaposition of the *Dct* promoter and *lacZ* coding region may combine elements that act together to result in ectopic expression.

Presumed melanoblasts were identified in embryos heterozygous for a null allele created by insertion of the lacZ reporter into the first exon of *Kit* (Bernex *et al.*, 1996). However, mice which lack one functional copy of *Kit* do not exhibit normal melanocyte development and these animals cannot give an accurate representation of melanoblasts in wild-type mice. Mast cells in the skin also express *Kit*, and these cells must be distinguished from cells of the melanoblast lineage. Because *Dct* is not expressed in mast cells, our study provides a much clearer overview of the role of Kit in the development of the melanocyte lineage in both wild-type and mutant embryos.

The $Kit^{W\text{-}v}$ Mutation

Intracellular signalling by Kit occurs upon homodimerisation induced by binding of a dimer of Mgf (Philo *et al.*, 1996). If it is assumed that mutant and wild-type heterodimers form as readily as (and are as stable as) homodimers, and that the kinase activities of heterodimers and mutant homodimers are similar, then the signalling activity of heterozygous melanoblasts will be at least 25% that seen in wild-type cells. The $Kit^{W\text{-}v}$ mutation reduces kinase activity in $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ mast cells to approximately 80–90% of the levels observed in +/+ or $Kit^{W\text{-}v}$/+ cells, although the abundance of $Kit^{W\text{-}v}$ transcripts and the level of protein expressed at the cell surface are normal (Nocka *et al.*, 1990). These authors do not report a difference between the kinase activities of +/+ or $Kit^{W\text{-}v}$/+ cells, although they clearly see a decrease in mast cell proliferation in heterozygotes. Similarly, our results show that the number of surviving melanoblasts is very much higher when only wild-type receptor homodimers are present compared to the receptors present on $Kit^{W\text{-}v}$/+ melanoblasts.

Kit Functions as a Survival Signal

Homozygous $Kit^{W\text{-}v}$ embryos at E10.5 clearly have melanoblasts, which have migrated away from the neural crest and lie in a pattern similar to those in wild-type embryos. At E11.5 only very few melanoblasts are visible in the

$Kit^{W\text{-}v}/Kit^{W\text{-}v}$ embryo; at later gestational stages none can be detected. Virtually all the labelled cells present at E10.5 disappear over the next 24 h, clearly demonstrating a role for the Kit receptor in melanoblast survival and confirming previous findings (Steel *et al.*, 1992).

Does Kit Function as a Proliferation Signal?

The number of melanoblasts in heterozygous embryos is greatly reduced with respect to wild type. This reduction in number presumably accounts for the overall dilution in coat colour (in addition to the head and belly spots) observed in the adult heterozygote, although the drop in melanoblast count is more dramatic than might have been predicted from the slight coat dilution observed. Our experiments demonstrate that Kit signalling is involved in proliferation beyond E12.5, as the difference in number of stained cells between the heterozygous and the wild-type embryos increases with embryonic age.

What can account for the reduced melanoblast numbers? Each melanoblast in the heterozygote has the same cell surface complement of Kit receptor (at least 25% that of wild-type cells) and presumably each is capable of receiving the same signal. If programmed cell death (PCD) in the absence of a survival factor is a stochastic process, in which the balance of probabilities between the death versus the survival pathways is determined by overall signalling input, then the reduced signalling in heterozygous cells may shift the balance in favour of death. On the other hand, if PCD is an all-or-nothing phenomenon, then the reduction in heterozygous melanoblast numbers can only be accounted for by reduced proliferation. Ultimately, the proliferative response of any cell to a signal is likely to be mediated by a combination of increased cell division and decreased PCD.

Does Kit Function in Melanocyte Migration?

The ligand of Kit, Mgf, exists in two forms, soluble and membrane bound, which may have distinct effects on Kit-expressing cells. Wehrle-Haller and Weston (1995) looked at the distribution of melanocyte precursors in two different mutants—Mgf^{Sl} (a null allele) and $Mgf^{Sl\text{-}d}$ (which produces the soluble form only). They suggest that soluble Mgf is required for the dispersal of melanocyte precursors onto their migration pathway or for their initial survival in the migration staging area (MSA, the region between the neural tube, the somite, and the overlying epithelium where neural crest cells are postulated to reside transiently after their segregation from the neural epithelium). They suggest that membrane-bound Mgf, on the other hand, is necessary for the survival of melanocyte precursors in the embryonic dermis once they have left the MSA. In our transgenic wild-type embryos we cannot identify melanoblasts accumulating in a region corresponding to the MSA, despite detecting transgene expression in the mesenchyme of embryos of 10 gestational days. If migrating melanoblasts do reside at the MSA they must do so only very transiently. We see no difference in the distribution of XGal-labelled cells in the mesenchyme lateral to the neural tube when comparing early +/+, $Kit^{W\text{-}v}/+$, and $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ embryos.

Our data do not show a direct role for Kit in the migration of melanoblasts, since the relative distributions of stained cells are the same in homozygous, heterozygous, and wild-type embryos at earlier stages of development (when melanoblasts are still visible in the homozygote) and in the heterozygous and wild-type embryos at the later stages. The regulation of growth or proliferation by Mgf is less efficient than regulation of adhesion to fibronectin and consequently cell migration (Kinashi and Springer, 1994). It is likely that high concentrations of Mgf, or high levels of Kit signalling, allow both proliferation and migration of melanoblasts, whilst at lower levels proliferation, but not migration, is compromised. With even lower (or zero) Mgf/Kit signalling, cells can neither proliferate nor migrate. The $Kit^{W\text{-}v}$ allele used in this study substantially reduces (without abolishing) tyrosine kinase activity relative to the wild-type receptor. It is conceivable that the residual kinase activity in homozygous mutant cells permits migration but is insufficient for significant proliferation. In heterozygous animals the kinase activity gives some, but reduced, proliferation, and migration is relatively unaffected. Although we find no direct evidence for an effect on the dispersal of melanocyte precursors, we cannot rule out the possibility that the Mgf/Kit interaction is important for the dispersal of melanoblasts via differential effects on proliferation versus migration, and we conclude that the Mgf/Kit interaction in early $Kit^{W\text{-}v}$ mutant embryos is primarily required for the survival of melanoblasts.

In summary, these transgenic mice provide a powerful system for observing melanocyte biology and will be extremely useful in elucidating the effects of a variety of coat colour mutations during embryogenesis and into postnatal life. Furthermore, it will now be possible to use cells from transgenic embryos as lineage markers to observe melanoblasts in chimaeric or grafted mouse embryos, just as the chick/quail chimaera system that has proved so effective for the study of avian neural crest cells.

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REFERENCES

Bernex, F., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C., and Panthier, J.-J. (1996). Spatial and temporal patterns of *c-kit*-expressing cells in $W^{lacZ}/+$ and W^{lacZ}/W^{lacZ} mouse embryos. *Development* **122,** 3023–3033.

Budd, P. S., and Jackson, I. J. (1995). Structure of the mouse tyrosinase-related protein-2/dopachrome tautomerase (*Tyrp2*/*Dct*) gene and sequence of two novel slaty alleles. *Genomics* **29,** 35–43.

Cable, J., Jackson, I. J., and Steel, K. P. (1995). Mutations at the *W* locus affect survival of neural crest-derived melanocytes in the mouse. *Mech. Dev.* **50,** 139–150.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., and Bernstein, A. (1988). The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* **335,** 88–89.

Copeland, N. G., Gilbert, G. J., Cho, B. C., Donovan, P. C., Jenkins, M. A., Cosman, D., Anderson, D., Lyman, S. D., and Williams, E. D. (1990). Mast cell growth factor maps near the *Sl* locus and is structurally altered in a number of *Steel* alleles. *Cell* **63,** 175–183.

Erickson, C. A. (1993). From the crest to the periphery: Control of pigment cell migration and lineage segregation. *Pigment Cell Res.* **6,** 336–347.

Geissler, E. N., Ryan, M. A., and Housman, D. E. (1988). The dominant-white spotting (*W*) locus of the mouse encodes the *c-kit* proto-oncogene. *Cell* **55,** 185–192.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241,** 42–52.

Huang, E., Nocka, K., Beier, D. R., Chu, T., Buck, J., Lahm, H.-W., Leder, P., and Besmer, P. (1990). The haematopoietic growth factor KL is encoded by the *Steel* locus and is the ligand for the *c-kit* factor, the product of the *W* locus. *Cell* **63,** 223–233.

Jackson, I. J., Chambers, D. M., Tsukamoto, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Hearing, V. (1992). A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse *slaty* locus. *EMBO J.* **11,** 527–535.

Kaufman, M. H. (1992). ''The Atlas of Mouse Development.'' Academic Press, London.

Kinashi, T., and Springer, T. A. (1994). Steel factor and c-kit regulate cell-matrix adhesion. *Blood* **83,** 1033–1038.

Kobayashi, T., Urabe, K., Winder, A., Jimenez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., Garcia-Borron, J. C., and Hearing, V. J. (1994). Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J.* **13,** 5818–5825.

Langtimm-Sedlak, C. J., Schroeder, B., Saskowski, J. L., Carnahan, J. F., and Sieber-Blum, M. (1996). Multiple actions of stem cell factor in neural crest cell differentiation *in vitro*. *Dev. Biol.* **174,** 345–359.

Lowings, P., Yavuser, U., and Goding, C. R. (1992). Positive and negative elements regulate a melanocyte-specific promoter. *Mol. Cell. Biol.* **12,** 3653–3662.

Mayer, T. C., and Green, M. C. (1968). An experimental analysis of the pigment defect caused by mutations at the *W* and *Sl* loci in mice. *Dev. Biol.* **18,** 62–75.

Morrison-Graham, K., and Weston, J. A. (1993). Transient steel factor dependence by neural crest-derived melanocyte precursors. *Dev. Biol.* **159,** 346–352.

Murphy, M., Reid, K., Williams, D. E., Lyman, S. D., and Bartlett, P. F. (1992). Steel factor is required for maintenance, but not differentiation, of melanocyte precursors in the neural crest. *Dev. Biol.* **153,** 398–401.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S.-I., Kunisada, T., Era, T., Sakakura, T., and Nishikawa, S.-I. (1991). *In utero* manipulation of coat colour formation by a monoclonal anti-c-kit antibody: Two distinct waves of c-kit-dependency during melanocyte development. *EMBO J.* **10,** 2111–2118.

Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., and Besmer, P. (1989). Expression of *c-kit* gene products in known cellular targets of *W* mutations in normal and *W* mutant mice—Evidence for an impaired c-kit kinase in mutant mice. *Genes Dev.* **3,** 816–826.

Nocka, K., Tan, J. C., Chiu, E., Chu, T. Y., Ray, P., Traktman, P., and Besmer, P. (1990). Molecular bases of dominant negative and loss of function mutations at the murine *c-kit*/white spotting locus: W^{37}, W^{v}, W^{41} and *W*. *EMBO J.* **9,** 1805–1813.

Pavan, W. J., and Tilghman, S. M. (1994). Piebald lethal (s^{l}) acts early to disrupt the development of neural crest-derived melanocytes. *Proc. Natl. Acad. Sci. USA* **91,** 7159–7163.

Philo, J. S., Wen, J., Wypych, J., Schwartz, M. G., Mendiaz, E. A., and Langley, K. E. (1996). Human stem cell factor dimer forms a complex with two molecules of the extracellular domain of its receptor, Kit. *J. Biol. Chem.* **271,** 6895–6902.

Reid, K., Nishikawa, S.-I., Bartlett, P. F., and Murphy, M. (1995). Steel factor directs melanocyte development *in vitro* through selective regulation of the number of c-kit+ progenitors. *Dev. Biol.* **169,** 568–579.

Reith, A. D., Rottapel, R., Giddens, E., Brady, C., Forrester, L., and Bernstein, A. (1990). *W* mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. *Genes Dev.* **4,** 390–400.

Steel, K. P., Davidson, D. R., and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* **115,** 1111–1119.

Tief, K., Hahne, M., Schmidt, A., and Beermann, F. (1996a). Tyrosinase, the key enzyme in melanin synthesis, is expressed in murine brain. *Eur. J. Biochem.* **241,** 12–16.

Tief, K., Schmidt, A., Aguzzi, A., and Beermann, F. (1996b). Tyrosinase is a new marker for cell populations in the mouse neural tube. *Dev. Dyn.* **205,** 445–456.

Wehrle-Haller, B., and Weston, J. A. (1995). Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. *Development* **121,** 731–742.

Williams, D. E., Eisenman, J., Baird, A., Ruach, C., Van Ness, K., March, C. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G. S., Cosman, D., and Lyman, S. D. (1990). Identification of a ligand for the *c-kit* proto-oncogene. *Cell* **63,** 167–174.

Yoshida, H., Kunisada, T., Kusakabe, M., Nishikawa, S., and Nishikawa, S.-I. (1996). Distinct stages of melanocyte differentiation revealed by analysis of non-uniform pigmentation patterns. *Development* **122,** 1207–1214.

Yoshida, H., Nishikawa, S.-I., Okamura, H., Sakakura, T., and Kusakabe, M. (1993). The role of *c-kit* proto-oncogene during melanocyte development in mouse: *In vivo* approach by *in utero* microinjection of anti-c-kit antibody. *Dev. Growth Differ.* **35,** 209–220.

Zsebo, K. M., Williams, D. A., Geissler, E. N., Broudy, V. C., Martin, F. H., Atkins, H. L., Hsu, R.-Y., Birkett, N. C., Okino, K. H., Murdock, D. C., Jacobsen, F. W., Langley, K. E., Smith, K. A., Takeishi, T., Cattanach, B. M., Galli, S. J., and Suggs, S. V. (1990). Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63,** 213–224.

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